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Studies on the biochemical and antibiotic patterns of the genus Citrobacter

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STUDIES ON THE BIOCHEMICAL AND ANTIBIOTIC PATTERNS OF THE GENUS *CITROBACTER*

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INTRODUCTION AND HISTORICAL BACKGROUND

The genus Citrobacter was proposed by Werkman and Gillen (1932) to include those Gram-negative enteric bacilli that were not typically Escherichia coli or Aerobacter aerogenes. Because of the ability of those "coli-aerogene intermediates" to utilize citrate as a sole source of carbon and to produce trimethylene glycol, these authors suggested the name Citrobacter for the group, and placed the 15 isolates they studied in seven species: C. freundii (type species), C. album, C. anindolicum, C. decolorans, C. diversum, C. glycologenes, and C. intermedium.

This suggestion by Werkman and Gillen was not accepted by a number of investigators including Yale (1939) and Vaughn and Levine (1942) who transferred C. freundii and C. intermedium, respectively, to the genus Escherichia.

A closely related group, the Bethesda-Ballerup group, was found by West and Edwards (1954) to resemble, biochemcially and serologically, E. freundii. Based chiefly on this study, the International Enterobacteriaceae Subcommittee recognized in 1958 the genus Citrobacter and accepted the designation of C. freundii (Braak) as its type species. A complete discussion of the history of this genus and related organisms appears in Edwards and Ewing (1972). According to these authors, Citrobacter is a member of the tribe Salmonellae and conforms to the following definition:

> "The genus *Citrobacter* is composed of motile bacteria that conform to the definitions of the family Enterobacteriaceae and the tribe Salmonellae. Lysine is not decarboxylated and less than 20% of strains possess ornithine decarboxylase. Urease is produced by a majority of cultures, but the reactions are weak. Growth occurs in medium that contains potassium cyanide and acid is produced in Jordan's tartrate medium. Dulcitol and cellobiose are fermented rapidly by the majority of the cultures. Lactose is utilized but the reactions are frequently delayed. The type species is *Citrobacter freundii* (Braak) Werkman and Gillen." (p. 276)

Today, three species are recognized in the genus: *C. freundii*, *C. amalonaticus*, and *C. diversus*. *C. freundii* is known to occur as three varieties: *C. freundii* H_2S +, IND -, *C. freundii* H_2S +, IND +, and *C. freundii* H_2S -, IND -.

It should be noted at this point that a suggestion was made (Young et al, 1974) to transfer *C. diversus* to the newly-created genus, *Levinea*. Although this suggestion has not been accepted officially, some authors refer to the organism as *C. diversus-Levinea*. However, for the purpose of this study, it will be referred to as *C. diversus*.

The role of *Citrobacter* in disease has not been investigated thoroughly. The organism is a normal inhabitant of the human intestine, being as common as *Escherichia coli* in infants. With age, the child's intestine begins to show a greater ratio of *E. coli* to *Citrobacter* (Nahhas, personal communication). *Citrobacter* has been reported as a cause of urinary and respiratory tract infections, especially in

children. Hodges et al (1978), however, believe that in most cases, Citrobacter is found in a commensalistic relationship with the organism(s) causing the infection. Most patients from whom Citrobacter was cultured had underlying diseases or factors predisposing them to infection. Outbreaks of neonatal meningitis in hospital maternity wards and nurseries have been attributed to contamination of nurses' hands and equipment by C. diversus (Anderson et al, 1981; Enzenauer et al, 1982). Osteomyelitis, neonatal diarrhea, neonatal septicemia, and brain abscesses caused by Citrobacter have also been reported (Barton and Walentik, 1982).

In January 1983, I began a study of this group with two objectives in mind: to study the biochemical and antibiogram characteristics of Citrobacter isolates from Stockton, California, and, if possible, to expand and update information on the biology of the genus.

MATERIALS AND METHODS

Bacterial Strains

A total of 109 strains of *Citrobacter* spp. were obtained between January 5, 1983 and February 29, 1984. These isolates were taken from clinical material recovered by the Microbiology Department at Dameron Hospital, Stockton, California. The specimens were one bronchial washing, one gall bladder, 97 stools, one umbilical cord, seven urine samples, and two wound. These were recovered from blood agar and/or EMB plates, and in the case of stools, *Yersinia* Selective Medium (YSM; Hana Co., Berkeley, CA). Most of the stool isolates were identified presumptively by their colonial morphology on YSM (red-colored colony) and reactions on Simmons' citrate (positive) and urea agar slants (negative or slightly positive). Single colonies were subcultured on tryptic soy agar slants and stored at 2-8°C for further biochemical and antibiogram studies.

Biochemical Studies

Identification of the isolates was based on the biochemical test results of the API 20E strip, a commercial kit purchased from Analytab Products, Inc., New York. These tests consist of beta-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), sodium citrate (CIT), hydrogen sulfide (H2S), urease (URE), tryptophan deaminase (TDA), indole (IND),

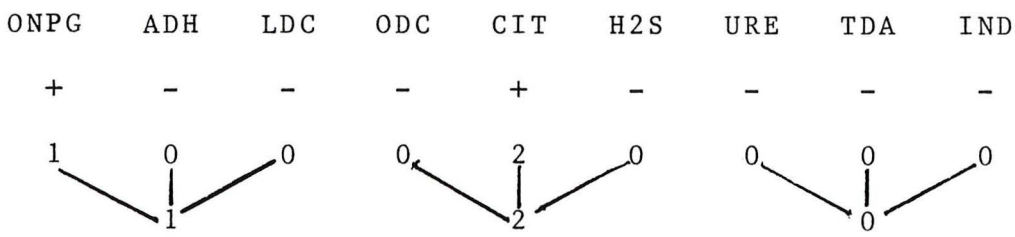
Vogues-Proskauer (VP), gelatin liquefaction (GEL), and nine carbohydrate fermentation tests: glucose (GLU), mannitol (MAN), inositol, (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY), and arabinose (ARA). Each strip was inoculated according to manufacturer's instructions. A small amount of each isolate was added to a test tube containing five ml of a 0.85% sterile saline solution (pH 5.5-7.0). Using a sterile pipette, the API 20E strip tubes were inoculated with the saline suspension. Tubes containing citrate, Vogues-Proskauer, and gelatin were filled to the rim with additional inoculum; sterile mineral oil was added to the tubes containing arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide, and urease to provide anaerobic conditions. Each API strip was incubated for 18-24 hours at 35^{o}C. Following incubation, positive results were noted and recorded. Positive results for the various tests are indicated in parentheses as follows: ONPG (yellow); ADH, LDC, ODC, URE (red or orange); CIT (turquoise or dark blue); H2S (black deposit); TDA (brown after adding one drop of 10% $FeCl_3$); IND (red ring after adding one drop Kovac's reagent); VP (red after adding one drop of 40% KOH and one drop of alpha-naphthol); GEL (diffusion of black pigment); GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA (yellow). The strips were reincubated for another 18-24 hours to note any delayed reactions.

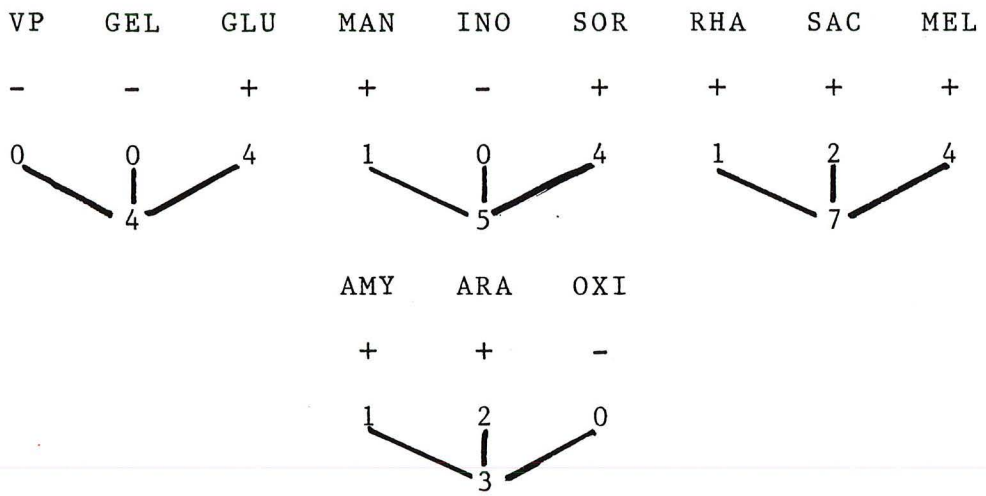
A seven-digit profile number was derived from the set

of results obtained from each strain. The oxidase (OXI) test made up the twenty-first test for this purpose. The 21 biochemical tests are divided into the following seven groups:

ONPG	ODC	URE	VP	MAN	RHA	AMY
ADH	CIT	TDA	GEL	INO	SAC	ARA
LDC	H2S	IND	GLU	SOR	MEL	OXI

Negative results were assigned a value of zero. Positive reactions were assigned a numerical value of one, two, or four, depending upon the location of the test within a group. A value of one was assigned for the first biochemical test in each group (ONPG, ODC, URE, VP, MAN, RHA, AMY). A value of two corresponds to the second biochemical test in each group (ADH, CIT, TDA, GEL, INO, SAC, ARA) and a value of four was assigned to the third test in each group (LDC, H2S, IND, GLU, SOR, MEL, OXI). A seven-digit profile number is obtained by adding the values within a group. An example follows for the results corresponding to the number 1204573:





A differential chart located in the API Profile for Enterobacteriaceae allows species identification on the basis of profile number. For example, profile number 1204573 keys out to C. freundii H_2S -, IND -.

In addition to the API 20E, citrate utilization was also studied in two other media: Simmons' citrate agar slants (Difco; sodium citrate 0.2%, magnesium sulfate 0.02%, ammonium dihydrogen phosphate 0.1%, dipotassium phosphate 0.1%, sodium chloride 0.5%, agar 1.5%, brom-thymol blue 0.008%) and Koser's citrate broth (Difco; sodium citrate 0.3%, sodium ammonium phosphate 0.15%, monobasic potassium phosphate 0.1%, magnesium sulfate 0.02%).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was conducted using the Kirby-Bauer standardized disc method (Bauer et al, 1966). Tryptic soy agar, however, was used instead of Mueller-Hinton medium. The plates were prepared by dissolving 40 gm of dehydrated medium in one liter of deionized water and dis-

pensing 66 ml into large screw-cap test tubes. After autoclaving, the tubes were allowed to cool and harden for storage. When needed, tubes were reautoclaved for five minutes and the media poured into sterile 150 mm plastic Petri dishes. Susceptibility testing procedures were conducted as follows: A small inoculum from a slant was introduced into a tryptic soy broth and incubated. The turbidity of actively growing cultures was then adjusted so as to obtain a suspension comparable to that of a barium chloride standard prepared by adding 0.5 ml of 0.048 M $BaCl_2$ (1.175% wt/vol, $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.36 N H_2SO_4 (1%, vol/vol). This is half the density of a McFarland No. 1 standard. Using a sterile cotton swab, a small amount of the suspension was streaked on the plates to insure even distribution of bacteria. The surface was allowed to dry for five to fifteen minutes and then antibiotic discs were placed on the surface of the plate using a 12-magazine-150 mm dispenser (Difco). The plates were incubated at 35°C for 18-24 hours and the diameter of each zone of inhibition was measured to the nearest mm. These diameters were compared to the standard chart (Table III) for interpretation of results.

All data was entered into the University of the Pacific (Stockton, CA) Burrough's B6700 large-systems computer by interactive terminal. The Statistical Package for the Social Sciences (SPSS) subroutine CROSSTABS was used to perform statistical analysis.

RESULTS

The computer-based profile system developed by Analytab Products for the API 20E places the 109 isolates into three species: C. freundii, 89 isolates (81.65%); C. diversus, 11 isolates (10.09%); and C. amalonaticus, nine isolates (8.26%)(Table I).

The majority, or 64 (71.91%) of the 89 C. freundii isolates were H_2S +, indole -, 17 (19.10%) were H_2S -, indole -, and eight (8.99%) were H_2S +, indole +. The distribution of the H_2S +, IND - strains indicates a predominance (58 isolates or 90.63%) in stools, five (7.81%) in urine, and one (1.56%) from a wound culture. Fifteen of the 17 isolates of H_2S -, indole - C. freundii (88.24%) were also of intestinal origin, one (5.88%) from the gall bladder, and one (5.88%) from a wound. Seven of the eight H_2S +, indole + C. freundii (87.50%) were stool isolates and one (12.50%) from a bronchial wash.

Of the 11 C. diversus isolates, eight (72.72%) were from stools, two (18.18%) from urine, and one (9.09%) from the umbilical cord stub of a newborn infant.

All nine isolates of C. amalonaticus were recovered from fecal cultures.

CITROBACTER FREUNDII

H_2S +, Indole - Biotype

Biochemical Activities

The biochemical activities of H_2S +, indole - C. freundii obtained in this study are shown in Table II. All 64 isolates were oxidase negative, failed to decarboxylate lysine, deaminate tryptophan, and produce urease and indole. The Vogues-Proskauer and gelatin liquefaction tests were also negative. All strains utilized Koser's citrate, produced H_2S after 48 hours (98% at 24 hours), and fermented glucose, lactose (ONPG), mannitol, rhamnose, and arabinose. Ninety-five percent of the isolates utilized Simmons' citrate and fermented sorbitol. Over 70% produced arginine dihydrolase after 48 hours (25% at 24 hours) and fermented sucrose and melibiose. Less than 40% decarboxylated ornithine, utilized citrate on the API strip, and fermented amygdalin. Inositol was fermented by only 9% of the isolates.

Antibiotic Susceptibility

The antibiogram for H_2S +, indole - C. freundii is shown in Table IV. All isolates were sensitive to cefamandole, cefotaxime, cefoperazone, gentamicin, amikacin, tobramycin, polymyxin B, naladixic acid, mezlocillin, and piperacillin. Over 90% were sensitive to carbenicillin, moxalactam, tetracycline, kanamycin, chloramphenicol, nitrofurantoin, and neomycin. Ninety-seven percent were resistant to

cephalothin while 95% were resistant to cefoxitin. Over 80% showed sensitivity to ampicillin, gantrisin, and trimethoprim-sulfamethoxazole (Bactrim-Septra).

H_2S +, Indole + Biotype

Biochemical Activities

The biochemical activities of the H_2S +, indole + *C. freundii* biotype are shown in Table II. All eight strains were oxidase, lysine decarboxylase, and tryptophan deaminase negative; negative reactions were also seen in urease, Vogues-Proskauer, and gelatin liquefaction tests. All eight isolates failed to ferment inositol. All isolates produced ornithine decarboxylase and utilized citrate on the API strip, Simmons' slant, and Koser's citrate broth; all strains produced H_2S and indole, and fermented glucose, lactose (ONPG), mannitol, sorbitol, rhamnose, sucrose, and arabinose. Seventy-five percent produced arginine dihydrolase after 48 hours (0% at 24 hours) and 88% fermented melibiose and amygdalin.

Antibiotic Susceptibility

The antibiotic susceptibility pattern of H_2S +, indole + *C. freundii* is shown in Table IV. All strains were resistant to cephalothin and cefoxitin, but sensitive to carbenicillin, cefamandole, cefotaxime, cefoperazone, moxalactam, tetracycline, gentamicin, kanamycin, amikacin, tobramycin, polymyxin B, chloramphenicol, gantrisin, trimethoprim-sulfamethoxazole (Bactrim-Septra), nitrofurantoin, naladixic acid, neomycin, mezlocillin, and piperacillin. Seventy-five percent showed a sensitivity to ampicillin.

H_2S -, Indole - Biotype

Biochemical Activities

The biochemical activities of the H_2S -, indole - *C. freundii* biotype are shown in Table II. All 17 isolates were oxidase, lysine decarboxylase, and tryptophan deaminase negative, and failed to produce H_2S, urease, and indole. Negative reactions were also seen in the Vogues-Proskauer and gelatin liquefaction tests. All isolates utilized Koser's citrate and fermented glucose, lactose (ONPG), mannitol, sorbitol, rhamnose, and arabinose. Inositol was not fermented. Over 90% of strains utilized Simmons' citrate and fermented sucrose, and 71% produced arginine dihydrolase after 48 hours (6% at 24 hours). Eighty-two percent fermented melibiose in 48 hours (76% at 24 hours). Less than 36% decarboxylated ornithine and utilized citrate on the API strip; amygdalin was fermented by only 24%.

Antibiotic Susceptibility

The antibiotic susceptibility pattern for H_2S -, indole - *C. freundii* is shown in Table IV. All isolates were resistant to cefoxitin, but sensitive to ampicillin, carbenicillin, cefamandole, cefotaxime, cefoperazone, moxalactam, tetracycline, gentamicin, kanamycin, amikacin, tobramycin, polymyxin B, chloramphenicol, gantrisin, trimethoprim-sulfamethoxazole (Bactrim-Septra), nitrofurantoin, naladixic acid, neomycin, mezlocillin, and piperacillin. Ninety-four

percent of isolates showed resistance to cephalothin.

CITROBACTER DIVERSUS

Biochemical Activities

The biochemical activities of C. diversus strains obtained in this study are shown in Table II. All 11 strains of C. diversus were oxidase negative, failed to decarboxylate lysine, produce H_2S, urease, and tryptophan deaminase, and gave negative reactions in the Vogues-Proskauer and gelatin liquefaction tests. All isolates produced arginine dihydrolase at 48 hours (91% at 24 hours), decarboxylated ornithine, utilized citrate on the API strip, produced indole, and fermented glucose, lactose (ONPG), mannitol, sorbitol, rhamnose, and arabinose after 48 hours (91% at 24 hours). Ninety-one percent of the isolates fermented sucrose, 88% amygdalin, and 64% inositol (55% at 24 hours); only 36% of the strains fermented melibiose.

Antibiotic Susceptibility

The antibiotic pattern of C. diversus is shown in Table IV. All isolates showed a resistance to ampicillin and carbenicillin, but a sensitivity to cephalothin, cefamandole, cefoxitin, cefotaxime, cefoperazone, moxalactam, tetracycline, gentamicin, kanamycin, amikacin, tobramycin, polymyxin B, chloramphenicol, gantrisin, trimethoprim-sulfamethoxazole (Bactrim-Septra), nitrofurantoin, naladixic acid, neomycin, mezlocillin, and piperacillin.

CITROBACTER AMALONATICUS

Biochemical Activities

The biochemical activities of C. amalonaticus strains obtained in this study are shown in Table II. All nine C. amalonaticus isolates were oxidase negative, failed to decarboxylate lysine, produce H_2S, urease, and deaminate tryptophan ; the Vogues-Proskauer and gelatin liquefaction tests were also negative. All strains decarboxylated ornithine, produced indole, and fermented glucose, lactose (ONPG), mannitol, sorbitol after 48 hours (78% at 24 hours), rhamnose, and arabinose; Simmons' citrate and Koser's citrate also gave positive reactions. Over 70% of isolates utilized citrate on the API strip and fermented sucrose and amygdalin. Only one isolate (11%) produced arginine dihydrolase after 48 hours and no isolates showed inositol fermentation.

Antibiotic Susceptibility

The antibiotic susceptibility pattern for C. amalonaticus is shown in Table IV. All isolates were sensitive to ampicillin, cephalothin, cefamandole, cefoxitin, cefotaxime, cefoperazone, moxalactam, tetracycline, gentamicin, kanamycin, amikacin, tobramycin, polymyxin B, chloramphenicol, gantrisin, trimethoprim-sulfamethoxazole (Bactrim-Septra), nitrofurantoin, naladixic acid, neomycin, mezlocillin, and piperacillin. Eighty-nine percent of the isolates showed sensitivity to carbenicillin.

DISCUSSION

The largest number (97) of Citrobacter isolates were recovered from stools. The Yersinia Selective Medium (YSM) was introduced in the mid-1970's for recovery of the enteric pathogen, Yersinia enterocolitica. YSM is not inhibitory to Citrobacter. The recovery of these species, therefore, was not unexpected since the intestine is one of the normal habitats for members of this genus. Citrobacter species can also be isolated from soil, water, food, and the gastrointestinal tracts of vertebrates other than humans.

Initially thought to be commensals or saprophytes, they have now been causally associated with opportunistic infections in children and adults (Barton and Walentik, 1982). These organisms have recently been isolated from bone, synovial fluid, peritoneal fluid, perinephric abscess, skin ulcers, ear, and vaginal discharges (Hodges et al, 1978). According to these researchers, extraintestinal infections by Citrobacter are seen mostly in those patients who are elderly, debilitated, and/or immunocompromised.

The 97 fecal isolates encountered in this study were from cultures submitted for "enteric pathogens." No enteric pathogens were recovered from these cultures. The 97 isolates were present with several other species and constitute a part of the normal intestinal flora. There is no

reason to attribute a pathogenic role to them in the intestinal tract.

The 12 isolates that were recovered from extrafecal sources included seven from urine, two from wounds, one from the umbilical cord of a newborn, one from a gall bladder, and one from a bronchial washing.

All seven urine isolates, five C. freundii and two C. diversus, were found in numbers exceeding 10^5/ml per culture, which definitely suggests urinary tract infections. The two wound cultures and the bronchial washing showed mixed infections, with C. freundii occurring in small numbers. Here, C. freundii may be assumed to be a contaminant. The isolate from the umbilical cord, a strain of C. diversus, was found in large numbers. Whether this finding represents a colonization of that site, or an actual infection was not determined. The gall bladder isolate, C. freundii, was found as a mixed culture with Klebsiella pneumoniae, both occurring in large numbers. Since the gall bladder is normally a sterile area, the presence of the two organisms represents a true infection. It may be concluded that the present study suggests at least a definite pathogenic role for Citrobacter freundii and C. diversus in urinary tract and gall bladder infections.

A large number of biochemical tests were performed in this study to ascertain proper identification. Tables V, VI, and VII compare the results of these tests with those of

Ewing and Davis (1971, 1972) and Edwards and Ewing (1972). These authors did not, however, distinguish among the three biochemical types of C. freundii.

It should be noted that certain tests on the API 20E strip do not correlate well with tests in the conventional media used in the above studies (for example, citrate and urease); however, when the citrate test was performed using conventional media, the results were similar to those of previous studies. Aldredge et al (1978) performed a study that compared the API 20E and Micro-ID (General Diagnostics, Morris Plains, NJ), another rapid multi-test biochemical identification kit, with conventional media. He found that discrepancies could occur between either the API 20E or Micro-ID kits and conventional media. It was found that, in a sample of 144 Citrobacter isolates, the Micro-ID misidentified four isolates and the API 20E, nine; only one was misidentified by conventional media. The authors hypothesized that the discrepancy in test results may be due to differing concentrations of reactants (in the citrate test, Koser's citrate uses 0.3% sodium citrate, Simmons' citrate contains 0.2% sodium citrate, and API 20E contains 0.8% sodium citrate) or that sensitivities of the substrate/reagent/inert compounds concentrations caused certain tests to have a tendency to emphasize either a positive or a negative reaction. It is possible, for example, that further incubation of the API 20E, for 72 or 96 hours, may produce a higher percentage

of positive reactions for citrate.

Identification of a species does not depend on any single test but rather a battery of tests. Computer-based identification schemes, such as the API 20E kit, take this fact into consideration and permit proper identification based on several profile numbers for each species.

When Edwards and Ewing carried out their studies on Citrobacter freundii, they apparently ignored any significance of H_2S and indole reactions, and worked on the assumption that there were no subspecies of C. freundii. However, today we know that there are at least three biochemical types (subspecies) of C. freundii based on their ability to produce H_2S and indole (or lack thereof).

Table II indicates that there is only one major difference between C. diversus and C. amalonaticus; arginine dihydrolase is produced by the former but not the latter. In the present study, inositol was fermented by 64% (55% at 24 hours) of C. diversus, but not by any of the strains of C. amalonaticus. The studies of Ewing and Davis (Table VI) and those of Edwards and Ewing (Table VII), however, show negative reactions for inositol by their strains of C. diversus and C. amalonaticus (in C. amalonaticus, 1.6% fermented inositol). This discrepancy again may be accounted for by the use of a commercial kit in the present study instead of conventional media. The point to be emphasized is that there is one test (out of 20) that distinguishes C. diversus from

C. amalonaticus.

The battery of tests used by Werkman and Gillen (1932) was relatively extensive and consisted of 37 substrates, including ammonium chloride, catalase, citrate, gelatinase, hydrogen sulfide, indole, litmus milk, methyl red, nitrate reduction, urease, Vogues-Proskauer, and carbohydrates: aesculin, amygdalin, arabinose, dextrin, dulcitol, erythritol, glucose, galactose, glycerol, glycogen, inositol, inulin, lactose, levulose, maltose, mannitol, mannose, melezitose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, and xylose. Fourteen of these 37 tests are represented on the API 20E strip. Table X lists and compares the reactions of the seven "species" of Werkman and Gillen with the Dameron Hospital isolates.

We should remember, however, that the description of the seven "species" was based on a total of 15 cultures (the number of each culture tested appears in parentheses next to the "species" in Table X). C. diversum of Werkman and Gillen differs from C. diversus by its ability to produce H_2S. C. amalonaticus does not match any of the Werkman and Gillen species. The only H_2S negative "species" is C. glycologenes, but this strain is also indole negative. Werkman and Gillen (1932) considered C. intermedium (deposited by Werkman and Gillen as ATCC 6750 in the American Type Culture Collection, Rockville, MD) as a non-H_2S-producer. The ori-

ginal description "appears to be in error" according to the twelfth edition (1976) of Catalogue of Strains I of the American Type Culture Collection, page 42. The only conclusion that can be made is that all of the "species" of Werkman and Gillen, except for the two isolates of C. decolorans, are probably Citrobacter freundii. Only two strains of the "species" described by Werkman and Gillen are available for study (ATCC strains 6750 and 8090) and both are recognized today as Citrobacter freundii (H_2S +, indole - biochemical type).

Based on the biochemical results of this study, the generic definition of Citrobacter may be emended as follows:

> The genus Citrobacter is composed of motile bacteria that conform to the definitions of the family Enterobacteriaceae. Lysine decarboxylase is not produced; ornithine decarboxylase and arginine dihydrolase production are variable. Urease is produced by a majority of cultures, but the reactions are weak. Growth occurs in medium that contains potassium cyanide and acid is produced in Jordan's tartrate medium. Dulcitol, cellobiose, mannitol, rhamnose, and arabinose are fermented. Lactose is utilized but the reactions frequently are delayed; however, orthonitrophenol-beta-D-galactosidase (ONPG) is positive. Oxidase, Vogues-Proskauer, and gelatinase tests are negative. The type species is Citrobacter freundii (Braak) Werkman and Gillen.

The use of antibiograms as an aid in the identification of bacteria is not new. It has been used to identify certain anaerobes, especially members of Bacteroides, Peptostreptococcus, Peptococcus, and Fusobacterium. It has also

been applied to Pseudomonas, and recently, the Klebsiella-Enterobacter-Serratia group (Hall, 1976). The basis for the use of an antibiogram in the identification of a bacterial agent is that certain genera or species are innately resistant to certain antibiotics; for example, all members of the genus Proteus are resistant to polymyxin B. Klebsiella pneumoniae and K. oxytoca are resistant to carbenicillin but sensitive to cephalothin, in contrast with members of the genera Serratia and Enterobacter, which are sensitive to the former but resistant to the latter (Darland, 1975; Nahhas, personal communication).

It is a common practice among bacteriologists to accept a trait as a major characteristic of a genus or a species if 90% or more of the strains exhibit this trait. This assumption, which works well for biochemical activities, may be applied, in a modified manner, to antibiotic patterns provided that antibiotic susceptibility studies are conducted under the same standardized conditions.

It should be pointed out that resistance of an organism to an antibiotic may be an innate characteristic of a genus as indicated above. Susceptibility, however, may change over a period of time as a result of mutations or plasmid-mediated transfer.

Using 90% as a guideline, one may make the conclusion that, as far as Citrobacter is concerned, its three species may be distinguished (chi square, α=0.05, Table IV) by a

pattern that involves the antibiotics ampicillin, carbenicillin, cephalothin, and cefoxitin. The other antibiotics showed uniform activity on all species. Because of possible differences in culture techniques and media batches, the interpretation of the data was somewhat flexible. Table III indicates that a difference of one mm in the size of the zone of inhibition may make a difference between an organism being "fully susceptible" to an antibiotic or "intermediate" in susceptibility, or between intermediate susceptibility and resistance. Table IV shows only susceptibility and resistance; all isolates that showed a zone size indicating intermediate susceptibility were placed either in the "susceptible" group or the "resistant" group depending on how close the zone size was to the lower limit of susceptibility or the upper limit of resistance. This is a standard procedure accepted by clinical microbiologists and is based on the observations that differing lots of media, antibiotic susceptibility discs, and concentration of inoculum affect, to a certain degree, the size of the zone of inhibition.

Based on the data in Table IV and on statistical analysis, the following antibiotic susceptibility patterns can be formulated (Table VIII). Citrobacter freundii is characterized by a sensitivity to ampicillin and carbenicillin, but is resistant to cephalothin and cefoxitin. C. diversus is characterized by being resistant to ampicillin and carbenicillin, but sensitive to cephalothin and cefoxitin. C.

amalonaticus is characterized by a sensitivity to ampicillin, carbenicillin, cephalothin, and cefoxitin.

It is evident that all three biochemical types of C. freundii show a similar antibiogram, but that in the case of H_2S +, IND - and H_2S +, IND + C. freundii strains, there is a small degree of variability in its reactions to several antibiotics, especially ampicillin. Studies done by Austen et al (1981) and Sawai et al (1982) help to explain this problem. They have been able to characterize the presence of the enzyme cephalosporinase in C. freundii which breaks down cephalothin and cefoxitin. The "third generation" cephalosporins, represented in this study by cefotaxime, cefoperazone, and moxalactam, are protected from the cephalosporinase produced by the organism, and thus sensitivity to these drugs is seen. It was also found that some strains of C. freundii contained a plasmid that would allow the organism to produce a beta-lactamase (penicillinase) that would make the organism resistant to penicillin and ampicillin (Austen et al, 1981; Sawai et al, 1982). This beta-lactamase is also present in C. diversus, but to a significantly larger extent than in C. freundii. The "new" penicillins, such as mezlocillin and piperacillin, which have a protected beta-lactam ring, are effective against these beta-lactamase-producing Citrobacter.

An interesting study into the evolutionary divergence of enteric bacilli, such as Citrobacter freundii, has re-

sulted in a finding that may have a clinical significance (Blumenberg and Yanofsky, 1982). While studying the tryptophan operon regulatory region in enteric bacteria, these authors found that certain strains of C. freundii contained a plasmid (R factor) that was identical to one found in Escherichia coli. This factor carries the gene responsible for beta-lactamase (penicillinase) production that makes both C. freundii and E. coli resistant to ampicillin. Finding that Citrobacter and Escherichia are similar in their genetic makeup, Blumenberg and Yanofsky concluded that it was possible that Citrobacter was derived from an ancestral form of Escherichia.

With respect to the evolutionary relationship of Citrobacter to the family Enterobacteriaceae, most authorities, including Edwards and Ewing (1972), have placed Citrobacter in the tribe Salmonellae. It seems to the author that, biochemically (Table IX), Citrobacter is intermediate in its characteristics between members of the tribes Escherichiae and Salmonellae. Genetically, at least according to Blumenberg and Yanofsky (1982), Citrobacter is closer to the genus Escherichia than to the genera of the tribe Salmonellae.

SUMMARY

One hundred and nine strains of *Citrobacter* isolated from various clinical material at Dameron Hospital, Stockton, California between January 1983 and February 1984 were investigated. The organisms were identified as follows: *C. freundii* (81.65%), *C. diversus* (10.09%), and *C. amalonaticus* (8.26%). Biochemical types of *C. freundii* were also identified as follows: H_2S +, IND - *C. freundii* (71.91%), H_2S +, IND + *C. freundii* (8.99%), and H_2S -, IND - *C. freundii* (19.10%). Each of the species was investigated with reference to distribution in clinical material, biochemical activity, and antibiotic susceptibility. Comparisons between results obtained in this study and in other studies were made with respect to biochemical activities and antibiograms. It was found that *C. freundii* shows resistance to cephalothin and cefoxitin, *C. diversus* is resistant to ampicillin and carbenicillin, and *C. amalonaticus* shows sensitivity to all antibiotics tested. This is in agreement with other investigators.

Based on this study and available information in the literature, the genus *Citrobacter* is emended.

TABLE I

Distribution of *Citrobacter* Isolates in Clinical Material

Species	Bronchial Washing	Gall Bladder	Stools	Umbilical Cord	Urine	Wound	Total
C. freundii, H_2S +, IND -	0	0	58	0	5	1	64
C. freundii, H_2S +, IND +	1	0	7	0	0	0	8
C. freundii, H_2S -, IND -	0	1	15	0	0	1	17
C. diversus	0	0	8	1	2	0	11
C. amalonaticus	0	0	9	0	0	0	9
Total	1	1	97	1	7	2	109

TABLE II

Biochemical Reactions of *Citrobacter* Isolates
(% positive reactions)

Species	Hours	OXI	ADH	LDC	ODC	API CIT	SIM CIT	KOS CIT	H2S	URE	TDA	IND	VP	GEL
C. freundii, H_2S +, IND - (n=64)	24	0	25	0	30	25	95	100	98	0	0	0	0	0
	48	0	72	0	30	31	95	100	100	0	0	0	0	0
C. freundii, H_2S +, IND + (n=8)	24	0	0	0	100	100	100	100	100	0	0	100	0	0
	48	0	75	0	100	100	100	100	100	0	0	100	0	0
C. freundii, H_2S -, IND - (n=17)	24	0	6	0	35	18	97	100	0	0	0	0	0	0
	48	0	71	0	35	29	97	100	0	0	0	0	0	0
C. diversus (n=11)	24	0	91	0	100	100	---	---	0	0	0	100	0	0
	48	0	100	0	100	100	---	---	0	0	0	100	0	0
C. amalonaticus (n=9)	24	0	0	0	100	78	100	100	0	0	0	100	0	0
	48	0	0	0	100	89	100	100	0	0	0	100	0	0

TABLE II (cont.)

Biochemical Reactions of *Citrobacter* Isolates
(% positive reactions)

Species	Hours	GLU	ONPG	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
C. freundii, H_2S +, IND - (n=64)	24	100	100	100	9	95	100	75	78	34	100
	48	100	100	100	9	98	100	77	78	39	100
C. freundii, H_2S +, IND + (n=8)	24	100	100	100	0	100	100	100	88	88	100
	48	100	100	100	0	100	100	100	88	88	100
C. freundii, H_2S -, IND - (n=17)	24	100	100	100	0	100	100	94	76	24	100
	48	100	100	100	0	100	100	94	82	24	100
C. diversus (n=11)	24	100	100	100	55	100	100	91	36	82	91
	48	100	100	100	64	100	100	91	36	82	100
C. amalonaticus (n=9)	24	100	100	100	0	89	100	89	78	78	100
	48	100	100	100	0	100	100	89	100	78	100

TABLE III

Zone Diameter Interpretive Standards Chart
(Kirby-Bauer Technique)

Antibiotics	Disc Content (mcg)	Zone Diameter (to nearest mm) Resistant (mm or less)	Intermed.	Sensitive (mm or more)
Ampicillin (AM)	10	11	12-13	14
Carbenicillin (CB)	100	17	18-22	23
Cephalothin (CR)	30	14	15-17	18
Cefamandole (MA)	30	14	15-17	18
Cefoxitin (FOX)	30	14	15-17	18
Cefotaxime (CTX)	30	14	15-22	23
Cefoperazone (CFP)	75	14	15-20	21
Moxalactam (MOX)	30	14	15-22	23
Tetracycline (TE)	30	14	15-18	19
Gentamicin (GM)	10	12	13-14	15
Kanamycin (K)	30	13	14-17	18
Amikacin (AN)	30	14	15-16	17
Tobramycin (TM)	10	12	13-14	15
Polymyxin B (PB)	300 units	8	9-11	12
Chloramphenicol (C)	30	12	13-17	18
Gantrisin (G)	300	12	13-16	17
Trimethoprim-Sulfamethoxazole (Bactrim-Septra; SXT)	25	10	11-15	16
Nitrofurantoin (FD)	300	14	15-16	17
Naladixic Acid (NA)	30	13	14-18	19
Neomycin (N)	30	12	13-16	17
Mezlocillin (MZ)	75	14	15-17	18
Piperacillin (PIP)	100	14	15-17	18

TABLE IV

Antibiograms of *Citrobacter* Species
(% susceptible)

Species		AM*	CB*	CR**	MA	FOX**	CTX	CFP	MOX	TE	GM	K	AN	TB
C. freundii, H_2S +, IND − (n=64)	R	16	5	97	0	95	0	0	2	9	0	2	0	0
	S	84	95	3	100	5	100	100	98	91	100	98	100	100
C. freundii H_2S +, IND + (n=8)	R	25	0	100	0	100	0	0	0	0	0	0	0	0
	S	75	100	0	100	0	100	100	100	100	100	100	100	100
C. freundii, H_2S −, IND − (n=17)	R	0	0	94	0	100	0	0	0	0	0	0	0	0
	S	100	100	6	100	0	100	100	100	100	100	100	100	100
C. diversus (n=11)	R	100	100	0	0	0	0	0	0	0	0	0	0	0
	S	0	0	100	100	100	100	100	100	100	100	100	100	100
C. amalonaticus (n=9)	R	0	11	0	0	0	0	0	0	0	0	0	0	0
	S	100	89	100	100	100	100	100	100	100	100	100	100	100

* - Significant differences seen between *C. diversus* and other two species (chi square, $\alpha = 0.05$)

** - Significant differences seen between *C. freundii* and other two species (chi square, $\alpha = 0.05$)

TABLE IV (cont.)

Antibiograms of *Citrobacter* Species
(% susceptible)

Species		PB	C	G	SXT	FD	NA	N	MZ	PIP
C. freundii, H_2S +, IND - (n=64)	R	0	5	12	11	2	0	2	0	0
	S	100	95	88	89	98	100	98	100	100
C. freundii, H_2S +, IND + (n=8)	R	0	0	0	0	0	0	0	0	0
	S	100	100	100	100	100	100	100	100	100
C. freundii, H_2S -, IND - (n=17)	R	0	0	0	0	0	0	0	0	0
	S	100	100	100	100	100	100	100	100	100
C. diversus (n=11)	R	0	0	0	0	0	0	0	0	0
	S	100	100	100	100	100	100	100	100	100
C. amalonaticus (n=9)	R	0	0	0	0	0	0	0	0	0
	S	100	100	100	100	100	100	100	100	100

TABLE V

Comparison of Biochemical Tests for *C. freundii*
(% positive reactions)

Test	*C. freundii* H_2S +, IND −	*C. freundii* H_2S +, IND +	*C. freundii* H_2S −, IND −	Edwards & Ewing* (1972)
Hydrogen Sulfide	98 (100)**	100	100	87.3
Urease	0	0	0	69.4
Indole	0	100	0	6.7
Vogues-Proskauer	0	0	0	0.0
Simmons' Citrate	95	100	97	94.6
Gelatin	0	0	0	0.0
Lysine Decarboxylase	0	0	0	0.0
Ornithine Decarboxylase	30	100	35	20.0
Arginine Dihydrolase	25 (72)	0 (75)	6 (71)	77.4
Phenylalanine Deaminase (TDA)	0	0	0	0.0
Glucose	100	100	100	100.0
Lactose	100	100	100	89.1
Sucrose	75 (77)	100	94	24.7
Inositol	9	0	0	4.2

* - Study did not differentiate among subspecies.

** - Figures in parentheses indicate percentages of delayed reactions.

TABLE VI

Comparison of Biochemical Tests for *C. diversus*
(% positive reactions)

Test	*C. diversus*	Ewing & Davis (1971,1972)
Hydrogen Sulfide	0	0.0
Urease	0	77.9
Indole	100	98.6
Vogues-Proskauer	0	0.0
Simmons' Citrate	100*	99.6
Gelatin	0	0.0
Lysine Decarboxylase	0	0.0
Ornithine Decarboxylase	100	100.0
Arginine Dihydrolase	91 (100)**	82.6
Phenylalanine Deaminase (TDA)	0	0.0
Glucose	100	100.0
Lactose	100	100.0
Sucrose	91	16.8
Inositol	55 (64)	0.0

* - Test is based on the API 20E strip

** - Figures in parentheses indicate percentages of delayed reactions

TABLE VII

Comparison of Biochemical Tests for *C. amalonaticus* (% positive reactions)

Test	*C. amalonaticus*	Edwards & Ewing (1972)
Hydrogen Sulfide	0	.0
Urease	0	74.8
Indole	100	95.2
Vogues-Proskauer	0	0.0
Simmons' Citrate	100	98.9
Gelatin	0	0.0
Lysine Decarboxylase	0	0.0
Ornithine Decarboxylase	100	100.0
Arginine Dihydrolase	0 (11)*	22.3
Phenylalanine Deaminase (TDA)	0	0.0
Glucose	100	100.0
Lactose	100	100.0
Sucrose	89	50.5
Inositol	0	1.6

* - Figure in parentheses indicate percentage of delayed reactions.

TABLE VIII

Identification of *Citrobacter* Species
Based on Biochemical Activity and Antibiograms

Species	Simmons' Citrate	Indole	H_2S	Inositol	Ampicillin	Carbenicillin	Cefoxitin
C. freundii H_2S +, IND -	+	-	+	-	S	S	R
C. freundii H_2S +, IND +	+	+	+	-	S	S	R
C. freundii H_2S -, IND -	+	-	-	-	S	S	R
C. diversus	+	+	-	+/-	R	R	S
C. amalonaticus	+	+	-	-	S	S	S

TABLE IX

Comparison of Genera in Tribes Escherichiae and Salmonellae*

Genus	H_2S	Simmons' Citrate	Urease	Gelatin	LDC	ODC	ADH	SAC	INO
TRIBE ESCHERICHIAE									
Escherichia	-(+)**	-	-	-	+/-	+	-	+	-
Shigella	-	-	-	-	-	-/+	-	-	-
TRIBE SALMONELLAE									
Citrobacter	+/-	+	+/-	-	-	+/-	-/(+)#	+/-	-
Salmonella	+	-/(+)	-	-	-	+	-/+	-	-/+
Arizona	+	-	-	(+)	+	+	-	+/-	-

* - Based on information found in Martin and Washington (1980).

** - Some strains of Escherichia coli produce H_2S.

\# - Delayed reaction after 48-hour incubation.

TABLE X

Comparison of Biochemical Reactions of
Werkman and Gillen's *Citrobacter* Isolates and Those from Dameron Hospital

Species	CIT	H2S	IND	VP	GEL	GLU	ONPG	MAN	INO	SOR	RHA	SAC	AMY	ARA
WERKMAN AND GILLEN'S ISOLATES														
C. freundii (2)	+	+	-	-	-	+	+	+	+	+	+	+	-	+
C. album (2)	+	+	-	-	+	+	+	+		+	+	-	-	+
C. glycologenes (1)	+	-	-	-	-	+	+	+		+	+	-	-	-
C. intermedium (4)	+	+	-	-	-	+	+	+	-	+	+	-	-	+
C. decolorans (2)	+	+	+	-	+	+	+	+	+	+	+	+	-	+
C. diversum (2)	+	+	+	-	-	+	+	+	+	+	+	+	-	+
C. anindolicum (2)	+	+	-	-	-	+	+	+		+	+	+	-	+
DAMERON ISOLATES (Numbers represent % positive reactions of biochemical tests)														
C. freundii, H_2S +, IND - (64)	100	100	0	0	0	100	100	100	9	98	100	77	39	100
C. freundii, H_2S +, IND + (8)	100	100	100	0	0	100	100	100	0	100	100	94	88	100
C. freundii, H_2S -, IND - (17)	100	0	0	0	0	100	100	100	0	100	100	94	24	100
C. diversus (11)	100	0	100	0	0	100	100	100	64	100	100	91	82	100
C. amalonaticus (9)	100	0	100	0	0	100	100	100	0	100	100	89	78	100

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